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(71) Applicant (for all designated States except US): UMC  
UTRECHT HOLDING B.V. [NL/NL]; Jenalaan 18a,  
NL-3584 CK Utrecht (NL).

(72) Inventors; and

(75) Inventors/Applicants (for US only): HUIZINGA, Eric,  
Geert [NL/NL]; Appelgaarde 5, NL-3992 JA Houten (NL).  
DE GROOT, Philip, Gerrit [NL/NL]; J. van Woensel  
Kooylaan 3, NL-1411 JX Naarden (NL). TSUJI, Shizuko  
[JP/NL]; Lange Smeestraat 28/IV, NL-3511 PX Utrecht

(NL). ROMIJN, Roland, Antonius, Paulus [NL/NL];  
Vlagzwam 22, NL-4007 NM Tiel (NL). SCHIPHORST,  
Marion, Eveline [NL/NL]; Kapelstraat 106, NL-3572  
CP Utrecht (NL). SIXMA, Jan, Johannes [NL/NL];  
Koningslaan 58, NL-3583 GM Utrecht (NL). GROS,  
Piet [NL/NL]; Ingenhouszstraat 61, NL-3514 HV Utrecht  
(NL).

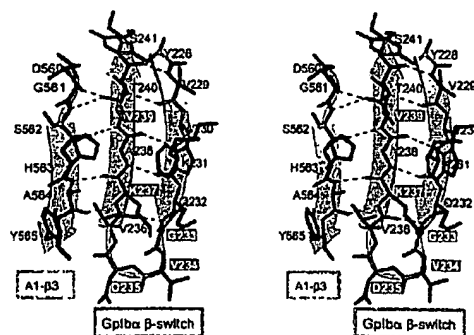
(74) Agent: PRINS, A.W.; Nieuwe Parklaan 97, NL-2587 BN  
Den Haag (NL).

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(54) Title: MODULATION OF PLATELET ADHESION BASED ON THE SURFACE EXPOSED BETA-SWITCH LOOP OF  
PLATELET GLYCOPROTEIN IB-ALPHA



(57) Abstract: The invention relates to the adhesion of platelet GpIba to strand b3 of domain A1 of von Willebrand factor (vWF), said strand b3 comprising amino acid residues at amino acid position 560-566 and/or a functional part or equivalent thereof, said platelet GpIbalpha, said GpIbalpha region comprising an amino acid sequence corresponding to a beta-switch loop of platelet GpIbalpha, comprising amino acid residues at amino acid position 227-242 and/or a functional part or equivalent thereof. The invention provides a method to interfere with said adhesion of blood platelets to vWF comprising modulating said adhesion. The invention further provides proteinaceous compounds, antibodies, medicaments and pharmaceutical compositions to that end. The invention also provides means and methods to increase platelet adhesion by topical application of a compound increasing platelet adhesion.



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**Title: MODULATION OF PLATELET ADHESION BASED ON THE SURFACE  
EXPOSED BETA-SWITCH LOOP OF PLATELET GLYCOPROTEIN IB-ALPHA**

The invention relates to the field of medicine, more specifically to the field of haematology, more specifically to platelet adhesion to von Willebrand Factor and the treatment of diseases in which platelet adhesion and blood clotting is involved.

5

Acute Coronary Syndromes (ACS) are a major cause of mortality in Western countries. The key event in an overwhelming number of these cases is platelet thrombus formation. Consequently there is strong interest in the development of safe and effective anti-thrombotic agents. Numerous agents  
10 have been developed that target the platelet activation cascade or prevent fibrinogen-mediated platelet-platelet aggregation (GPIIb/IIIa inhibitors). These approaches have produced some encouraging results. However, direct inhibition of the very first step in thrombus formation, namely formation of the initial platelet monolayer has received comparatively little attention to date.  
15 Platelet rolling over exposed subendothelium at sites of vascular injury is a crucial initiating step in haemostasis and thrombosis. This process depends critically on the interaction of platelet-receptor glycoprotein Ib $\alpha$  (GpIb $\alpha$ ) and plasma-protein von Willebrand Factor (vWF). GpIb $\alpha$  not only plays a role in the adhesion to vWF, but also triggers the platelet into an activated state.

20 This application has identified and characterized that a part of (GpIb $\alpha$ ) forms a flexible and surface exposed loop aa227-242, in this application designated as a "Beta-switch". This peptide, formerly supposed to be an  $\alpha$  chain, surprisingly, has been found to control platelet binding to von Willebrand factor (vWf) by controlling the binding of platelet GpIb $\alpha$ , aa227 to  
25 242 region to the Beta3 strand and its preceding turn region of the A1 region of vWF protein (aa560-566) to form a continuous Beta-sheet shared between the two molecules. In this application, the Beta3 strand, comprising aa562-566

and its preceding turn region of the A1 region of vWF protein comprising aa560-562, together will be named the Beta3 strand. Said binding to vWF is essential for subsequent platelet adhesion to the subendothelial layer or to damaged endothelial cells and the following platelet activation. Thus the flexible and surface exposed loop aa227-242, in this application designated as a "Beta-switch" and/or a functional derivative thereof, is an efficient controller of platelet adhesion and platelet activation and thrombus formation *in vivo*.

This application shows structures of the human GpIb $\alpha$  N-terminal domain, and its complex with human vWF domain A1. The structure of GpIb $\alpha$ , containing 8 leucine-rich repeats flanked by capping sequences characteristic of extra-cellular proteins with these repeats, wraps around one side of vWF-A1 providing a large contact area. Perturbing this *in vivo* modulated interaction explains four types of congenital bleeding disorders caused by mutations in either GpIb $\alpha$  or vWF-A1.

These findings provide a structural basis for understanding this critical interaction in platelet rolling, which is relevant for the development of novel anti-thrombotics. The ability to inhibit primary platelet adhesion potentially presents significant advantages over alternative thrombotic therapies because the former targets the very first step of platelet aggregation and thrombus formation.

Identification of the function of the peptide loop aa 227-242 of GpIb $\alpha$  as a Beta-switch and adhesion site to strand  $\beta$ 3 of domain A1 of vWF, enables a person skilled in the art to develop a method to interfere with platelet adhesion comprising modulating the adhesion of amino acid residues at amino acid position 560-566 of strand  $\beta$ 3 of domain A1 of von Willebrand factor (vWF), to amino acid residues at amino acid position 227-242 of a region of platelet GpIb $\alpha$ , said GpIb $\alpha$  region corresponding to a beta-switch loop of platelet GpIb $\alpha$ . In a preferred embodiment of the invention, said GpIb $\alpha$  region comprises amino acid residues at amino acid position 227-242.

In a more preferred embodiment, said GpIb $\alpha$  region comprises amino acid residues at amino acid position 200-300.

This interfering can be both a decrease or an increase of platelet adhesion. Decreasing the platelet adhesion will result in a decreased tendency  
5 of platelets to stick to vessel walls and damaged endothelium, a decreased thrombus formation, and dissolving of an existing thrombus. These features are very helpful for preventing and curing acute coronary syndromes, as well as other diseases wherein platelet adhesion and thrombus formation play a role.

10 Increasing the platelet adhesion will result in more effective stopping of bleeding from wounds or cuts and can result in a treatment for certain bleeding disorders.

Now that the specific site of adhesion between vWF and platelet GpIb $\alpha$  is known, it is clear to any person skilled in the art to select or make and test  
15 compounds that block this adhesion.

In one embodiment of the invention, the compound that interferes with the adhesion of said strand  $\beta$ 3 of vWF to a region of platelet GpIb $\alpha$  may comprise a chemical or proteinaceous compound capable of interfering with the binding of said platelet GpIb $\alpha$  peptide to said region of vWF. Said compound  
20 also comprises a fusion protein, at least containing part of amino acids 560-566 of strand  $\beta$ 3 of domain A1 of vWF, or of amino acids 227-242 of platelet GpIb $\alpha$ , or both.

In a preferred embodiment of the invention, said compound comprises an amino acid sequence corresponding to strand  $\beta$ 3 of domain A1 of vWF,  
25 comprising amino acid residues at amino acid position 560-566 and/or a functional part or equivalent thereof.

In another preferred embodiment of the invention, said compound comprises an amino acid sequence corresponding to a beta-switch loop of platelet GpIb $\alpha$ , comprising amino acid residues at amino acid position 227-242  
30 and/or a functional part or equivalent thereof. A functional part of a protein is

defined as a part which has the same properties in kind, not necessarily in amount. By properties of amino acid position 227-242 of platelet GpIb $\alpha$  is meant the flexible and surface exposed loop called the Beta-switch and the adhesion site of platelet GpIb $\alpha$  to strand  $\beta$ 3 of domain A1 of vWF. A functional equivalent of a protein is defined as a protein which has been altered such that the properties of said molecule are essentially the same in kind, not necessarily in amount. An equivalent can be provided in many ways, for instance through conservative amino acid substitution.

A person skilled in the art is well able to generate analogous compounds or derivatives of a protein. This can for instance be done through screening of a peptide library. Such an analogue has essentially the same properties of said protein in kind, not necessarily in amount. In this application, an equivalent also includes analogous compounds. Derivatives may include chemical modifications of biological molecules or equivalents thereof as defined above. Such chemical modifications may be introduced, for instance, to provide an (additional) group for coupling vWF and/or platelet GpIb $\alpha$  or to prolongue the circulation time in the body. A proteinaceous molecule comprises at least two amino-acids linked to each other with a peptide bond. It preferably further comprises additional amino-acids linked with peptide bonds. A proteinaceous molecule may comprise a modification such as typically added in post-translational modification reactions of proteins. Non-limiting examples are glycosylation and/or myristilisation. One or more of the amino acids may also comprise a chemical modification.

The adhesion of platelet GpIb $\alpha$  to vWF can also be interfered by antibodies specifically recognizing either the strand  $\beta$ 3 of domain A1 of vWF, comprising amino acid residues at amino acid position 560-566, or the beta-switch loop of platelet GpIb $\alpha$ , comprising amino acid residues at amino acid position 227-242, so in a preferred embodiment of the invention, said compound comprises an antibody and/or a functional part or equivalent thereof, specifically recognizing said region of platelet GpIb $\alpha$  and/or said

region of strand  $\beta 3$  of domain A1 of vWF. Abovementioned compounds can be administered to individuals for interfering with platelet adhesion and/or thrombus formation. It may be favorable to couple said compound to another compound, which is capable of prolonging the circulation time. Therefore, this application teaches the use of said compound either directly, and/or linked to another chemical and/or a proteinaceous compound, wherein said second compound modulates the circulation time of the first compound. Said second compound comprises any chemical or proteinaceous substance that is known in the art to extend the circulation time of said first compound. In another embodiment, said compound is used for the preparation of a medicament for the treatment or prevention of a disease in which platelet adhesion and/or thrombus formation is involved. Said medicament can for example be administered by parenteral inoculation, or by oral administration in the form of tablets or pills or as a potion. This medicament may be combined with other substances to optimize the action, for example by combination with a slow release compound, and the shelf-life of the medicament, for example by adding stabilizing agents, and to facilitate the administration.

In another embodiment, this application teaches a pharmaceutical composition for inhibiting platelet adhesion and/or thrombus formation comprising said compound further comprising a suitable carrier or solvent.

In yet another embodiment of the invention, said medicament or pharmaceutical composition is used to modulate platelet adhesion and/or thrombus formation.

Because this application teaches the specific adhesion sites of strand  $\beta 3$  of domain A1 of vWF, comprising amino acid residues at amino acid position 560-566 and of a beta-switch loop of platelet GpIb $\alpha$ , comprising amino acid residues at amino acid position 227-242, antibodies can be produced against these sites that interfere with platelet adhesion. Therefore, this application teaches an antibody and/or a functional part or equivalent thereof, specifically recognising a region of platelet GpIb $\alpha$  according to claim 1, capable

of interfering with platelet adhesion. And, in another embodiment, this application teaches an antibody and/or a functional part or equivalent thereof, specifically recognising strand  $\beta 3$  of domain A1 of vWF, capable of interfering with platelet adhesion. In yet another embodiment, this application teaches  
5 the use of said antibodies and/or a functional part or equivalent thereof for the preparation of a medicament for the treatment of a disease in which platelet adhesion and/or blood clotting is involved.

The amino acid sequence of said beta-switch loop of GpIb $\alpha$ , and the amino acid sequence of strand  $\beta 3$  of domain A1 of vWF can be built into a  
10 vector using standard techniques. Therefore, this application teaches a vector which is capable of effecting the expression of a peptide with an amino acid sequence corresponding to a beta-switch loop of platelet GpIb $\alpha$ , comprising amino acid residues at amino acid position 227-242 and/or a functional part or equivalent thereof, in an appropriate environment. And, in another  
15 embodiment, this application teaches a vector which is capable of effecting the expression of a peptide with an amino acid sequence corresponding to strand  $\beta 3$  of domain A1 of vWF, comprising amino acid residues at amino acid position 560-566 and/or a functional part or equivalent thereof, in an appropriate environment. Because this application teaches how to produce a  
20 medicament and/or a pharmaceutical composition and/or antibodies and/or vectors as mentioned above, it also teaches a method of treatment or prevention of a disease condition in which platelet adhesion is involved, comprising administering said medicament and/or said pharmaceutical composition and/or said antibody, and/or said vector.

25 As has been mentioned above, a peptide or a proteinaceous compound with an amino acid sequence corresponding to strand  $\beta 3$  of domain A1 of vWF, comprising amino acid residues at amino acid position 560-566 and/or a functional part or equivalent thereof, capable of binding platelet GpIb $\alpha$ , can be used to locally increase the adhesion of platelets and the  
30 formation of a thrombus. Therefore, this application also teaches a method to

enhance platelet adhesion comprising local application of a peptide or a proteinaceous compound with an amino acid sequence corresponding to strand  $\beta 3$  of domain A1 of vWF, comprising amino acid residues at amino acid position 560-566 and/or a functional part or equivalent thereof, capable of binding platelet GpIb $\alpha$ . Said compound may be bound to a matrix like for example a wound dressing, or a band aid, or may be combined with an ointment or a glue or another suitable carrier or solvent. In another embodiment of this invention, said peptide or a proteinaceous compound with an amino acid sequence corresponding to strand  $\beta 3$  of domain A1 of vWF, comprising amino acid residues at amino acid position 560-566 and/or a functional part or equivalent thereof, can be used for the manufacturing of a topical medicament for local enhancement of platelet adhesion and/or thrombus formation and for the manufacturing of a pharmaceutical composition for local enhancement of platelet adhesion and/or thrombus formation comprising a peptide or a proteinaceous compound with an amino acid sequence corresponding to strand  $\beta 3$  of domain A1 of vWF, comprising amino acid residues at amino acid position 560-566 and/or a functional part or equivalent thereof, and a suitable carrier or solvent. Therefore, this application also teaches such a pharmaceutical composition for local enhancement of platelet adhesion and/or thrombus formation comprising a peptide or a proteinaceous compound with an amino acid sequence corresponding to strand  $\beta 3$  of domain A1 of vWF, comprising amino acid residues at amino acid position 560-566 and/or a functional part or equivalent thereof, and a suitable carrier or solvent. This application also teaches providing said medicament and/or said pharmaceutical composition to an individual, for example suffering of a bleeding disorder, for example but not limited to von Willebrand disease type 2M, as a method of treatment or prevention of a disease condition in which platelet adhesion is decreased.

The invention is further explained in the following example.

**Example 1.**

**Insight into platelet rolling by crystal structures of Glycoprotein Iba and von Willebrand Factor A1 domain**

5           The interaction of platelet-receptor glycoprotein Iba (GpIba) and immobilised von Willebrand Factor (vWF) at sites of vascular damage mediates rolling of platelets <sup>1</sup>. Transient interactions of the platelet with vWF greatly reduces platelet velocity and prolongs the contact time with reactive components of the cell matrix, necessary for platelet-activation and integrin-mediated firm attachment. In rapidly flowing blood, this vWF mediated rolling is essential for platelet adhesion <sup>2</sup>.

          GpIba is the central component of a receptor complex consisting of glycoproteins Iba, Ib $\beta$ , IX and V. It anchors the complex to the cytoskeleton and contains the VWF-binding function in its ~290 N-terminal residues. The VWF-binding site is exposed well above the platelet surface, being connected to a ~45 nm long highly O-glycosylated stalk <sup>3</sup>. The ~250-kDa vWF protein forms large disulfide-bonded multimers with molecular weights of up to 10 MDa. It is found in plasma and the sub-endothelial cell matrix and is released from storage granules when platelets and endothelial cells are activated. A vWF-multimer acts as bridging ligand between platelets and the cell matrix, through collagen binding by its A3 domain and binding to GpIba by its A1 domain <sup>4</sup>.

          vWF and platelet GpIba coexist in the circulation and interact with each other when vWF has been bound to a surface. A disturbed balance with deleterious consequences is apparent in a number of congenital bleeding disorders <sup>5,6</sup>. In von Willebrand disease type 2M and the Bernard-Soulier syndrome loss-of-function mutations in the vWF-A1 domain and GpIba respectively, lead to reduced affinity and cause a bleeding tendency. In von

Willebrand disease type 2B and platelet type pseudo-von Willebrand disease, gain-of-function mutations in vWF and GpIb $\alpha$ , respectively, cause an increased affinity sufficient to support interaction between soluble vWF and platelets, leading to intravascular platelet clumping, reduced platelet counts, and a  
5 resulting bleeding tendency. Shear stress modulates the GpIb $\alpha$ -vWF affinity<sup>7</sup>; vigorous stirring can induce vWF-mediated platelet aggregation. In thrombosis, shear stress induced interaction of GpIb $\alpha$  and vWF occurs in arteries occluded by atherosclerotic plaque. The precise interactions between GpIb $\alpha$  and A1 of vWF, the molecular basis of the bleeding disorders and the  
10 mechanism of activation are poorly understood. In this paper we present crystal-structures of the N-terminal domain of GpIb $\alpha$  (residues 1-290) and its complex with the vWF-A1 domain (residues 498-705), revealing key interactions in these processes.

## 15 Structure of GpIb $\alpha$

The von Willebrand factor-binding domain of GpIb $\alpha$  displays an elongated curved shape (Figure 1A), that is typical for proteins containing leucine-rich repeats. The structure shows for the first time the flanking regions conserved among numerous extra-cellular proteins, including the other  
20 members of the GpIb-IX-V complex. The central region of the molecule consists of eight short leucine-rich repeats of which seven were predicted based on the amino-acid sequence. At the N-terminal flank the leucine-rich repeats are preceded by a 14-residue  $\beta$ -hairpin delimited by a conserved disulphide bond between Cys4 and Cys17. The tip of the  $\beta$ -hairpin, which we refer to as  $\beta$ -  
25 finger, protrudes from the protein surface and is disordered in one of the two molecules in the asymmetric unit. At the C-terminal flank, up to residue 267, the leucine-rich repeats are followed by a region containing a 9-residue  $\alpha$ -helix and four short  $3_{10}$ -helices. The subsequent anionic region of GpIb $\alpha$  was invisible in the electron-density and has not been modelled. Conserved  
30 cysteine residues in the C-terminal flank at positions 209 and 211 form

disulphide bonds to Cys248 and Cys264, respectively, stabilising the observed irregular fold. Residues 227 to 242 project from the concave face of the molecule forming a highly flexible loop that shows disorder in both molecules in the asymmetric unit. Sequence alignment of C-terminal flanking regions shows that the protruding loop is not a conserved feature in this domain-family. Remarkably, it is this flexible protruding loop that contains the gain-of-function mutations causing platelet-type pseudo-von Willebrand disease.

### Structure of the GpIb $\alpha$ -A1 complex

We solved the structure of GpIb $\alpha$  in complex with vWF-A1 using mutant proteins, related to platelet-type pseudo- and type 2B von Willebrand diseases, which increase the affinity of complex formation. Individually, mutations GpIb $\alpha$  M239V and vWF-A1 R543Q increase the affinity by a factor of 3.0 and 2.5, respectively. Together these mutations increase the affinity 5.7-fold yielding a K<sub>d</sub> of 5.8 nM (Biacore binding data are given in Table 2).

In the complex the A1 domain fits into the concave curve of GpIb $\alpha$  (Figure 1B), burying a solvent accessible surface of ~1,900 Å<sup>2</sup>. The N-terminal  $\beta$ -hairpin, the leucine-rich repeat region, and the C-terminal flank of GpIb $\alpha$  all interact with A1 and define an extended but discontinuous binding site that involves residues close to the top face and on the bottom-face of the A1 domain. As for the native structure of GpIb $\alpha$  we do not observe electron density for the anionic region of GpIb $\alpha$  in the complex. This is surprising in view of the role ascribed to the anionic region in vWF-binding<sup>8</sup>, but is consistent with our observation that the anionic region has no significant effect on K<sub>d</sub>'s (Table 2).

The flexible and surface-exposed loop 227-242 in GpIb $\alpha$ , which contains mutation M239V, undergoes a remarkable conformational change upon complex formation. In the complex this loop, that we call  $\beta$ -switch, forms a 16-residue  $\beta$ -hairpin that extends from residues 227 to 242. It aligns with strand  $\beta$ 3 of A1 (residues 562-566) thus forming a continuous  $\beta$ -sheet shared between the two molecules (Figure 1D). Interactions between the  $\beta$ -switch and

strand  $\beta 3$  of A1 are predominantly backbone-backbone in nature. The mutated residue Val239 of GpIb $\alpha$  is located in the  $\beta$ -switch strand that directly hydrogen bonds to  $\beta 3$  of A1. Its side chain has hydrophobic contacts with residues Phe199 and Phe201 of GpIb $\alpha$  and Tyr600 of helix  $\alpha 3$  in A1.

5           Residues of the GpIb $\alpha$  C-terminal flank and leucine-rich repeats lrr-8 to lrr-6 form a continuous surface that interacts tightly with A1 helix  $\alpha 3$  and loop  $\alpha 3\beta 4$  (Figure 1C). Glu596 and Lys599 of helix  $\alpha 3$  of A1 are indispensable for GpIb $\alpha$  binding<sup>9,10</sup> and are engaged in extensive interactions. From lrr-5 to lrr-1 the interactions between the molecules peter out. In fact, A1 interacts  
10 only with a rim of the leucine-rich repeat concave face made up of residues that are located in the first position of the  $\beta$ -strand or immediately N-terminal to the  $\beta$ -strand in lrr-4 to lrr-8. At the bottom, the N-terminal  $\beta$ -finger of GpIb $\alpha$  regains contact with A1 near its N- and C-terminus and contacts residues in loops  $\alpha 1\beta 2$ ,  $\beta 3\alpha 2$ , and  $\alpha 3\beta 4$ . Compared to the free structure of  
15 GpIb $\alpha$  the  $\beta$ -finger has made a small relative shift of 2 Å away from the A1 domain. Remarkably, residues Lys549, Trp550 and Arg571 are disordered at this interaction site showing reduced electron density for side-chains. The mutation R543Q in A1 is located 20 Å away from the interaction region with GpIb $\alpha$ . It is part of loop  $\alpha 1\beta 2$  that in other structures of A1 interacts with the  
20 N-terminal region of the A1 domain<sup>11</sup>.

### Molecular basis of congenital bleeding disorders

Figure 2A shows point mutations associated with the Bernard-Soulier syndrome and von Willebrand type 2M diseases, which strongly reduce  
25 the interaction between GpIb $\alpha$  and A1, yielding a loss of function. Mutations in GpIb $\alpha$ , L57F, C65R, L129P and a deletion of Leu179, occur at buried sites critical for the structural integrity of the leucine-rich repeats. Mutation C209S prevents the formation of a conserved disulphide bond in the C-terminal flanking region. Mutation A156V occurs in lrr-6 at the concave face of the  
30 protein. Replacement by valine at this site may not be tolerated because of

tight packing interactions with neighbouring residues (Ala156 is not solvent accessible) and could affect the conformation of nearby residue Lys159 and the  $\beta$ -switch that interact with A1.

Loss-of-function mutations in A1, detected in von Willebrand disease type 2M patients or identified by site-directed mutagenesis<sup>9,10,12-14</sup>, are numerous and scatter throughout the A1 domain. Interpretation of the site-directed mutagenesis data is complicated by differences in experimental procedures, i.e. the use single domain A1 vs. full-length vWF and the use of the artificial activators ristocetin and botrocetin. Sixteen loss-of-function mutations involve residues in or directly next to the GpIb $\alpha$  binding site, whereas 10 occur at buried positions inside the A1 domain, and are likely to disrupt the A1 structure or induce conformational changes to A1 incompatible with binding to GpIb $\alpha$ . The remaining 14 mutations are at the surface, but outside the observed GpIb $\alpha$  binding site.

In platelet-type pseudo-von Willebrand disease mutations in GpIb $\alpha$  enhance the interaction with A1 leading to a gain-of-function phenotype. The five known gain-of-function mutations, G233V and M239V detected in patients<sup>15,16</sup> and V234G, D235V and K237V identified by site-directed mutagenesis<sup>17,18</sup>, occur in the  $\beta$ -switch loop (see Figure 1D). Four of these mutations are known to stabilise  $\beta$ -hairpin structures<sup>19</sup> by introducing a C $\beta$ -branched residue in the strands (G233V, M239V and K237V) or a glycine residue in the tight turn (V234G). Our kinetic analysis shows that mutation M239V yields a three-fold increase in the association rate, and hardly affects the dissociation rate, which is consistent with stabilisation of the  $\beta$ -hairpin priming the mutant GpIb $\alpha$  for A1-binding. Unclear at this stage is how D235V, at the second position in the tight turn, induces  $\beta$ -hairpin formation and enhancement of the binding affinity. However, all five mutations support an indirect mechanism by influencing the conformation, because the side chains have either no or few direct contacts to A1 in the complex (see Figure 1D). Other valine substitutions

in this region, K231V, Q232V, A238V and T240V reduce binding affinity<sup>18</sup>. These may be explained in part by steric hindrance (Q232V and A238V) and loss of a hydrogen bond with Asp560 of A1 (T240V). Altogether, we may conclude that the conformational equilibrium of the  $\beta$ -switch of GpIb $\alpha$  is a  
5 critical factor in the precisely balanced affinity of the interacting partners.

The von Willebrand disease type 2B gain-of-function mutations in A1 cluster on one side of the central  $\beta$ -sheet at the bottom face of the domain (see Figure 2B). Type 2B mutation R543Q that we have used in the structure determination of the complex causes a 2.5 fold decrease in K<sub>d</sub> and affects both  
10 the association and dissociation rate. The wild-type A1 domain has a K<sub>d</sub> of 33 nM and binds GpIb $\alpha$  much stronger than multimeric vWF, for which we cannot detect any binding up to a concentration of 150 nM (data not shown). This indicates that important structural elements required for keeping A1 in a low affinity state are not present in the isolated domain used for crystallisation.  
15 Some of the type 2B mutations (K549A, W550C) are close to the interaction site with the  $\beta$ -finger of GpIb $\alpha$ , but most are far from the interaction site and appear involved in interactions with the N- and C-terminal flanking peptides of the A1 domain. Comparison of A1 (R543Q) of the GpIb $\alpha$  A1 complex with wt-A1 structures<sup>11,20</sup> shows several differences in the region of type 2B  
20 mutations. However, the interpretation of these differences is complicated by extensive crystal contacts in this area. Differences involve N-terminal residues 505-511 and C-terminal residues 694-696 and 700-703, including disulphide bond 509-695 that links the N- and C-terminal regions and shifts by about 2.5 Å. The conformation of the main chain of residues 544-551 is largely  
25 unchanged, but this loop appears more flexible in the complex with poorly resolved electron-density for the side-chains. The conformational differences observed could represent activation of the A1 domain towards GpIb $\alpha$  binding.

### Implications for rolling interactions

Tethering and rolling in platelet adhesion to sites of vascular damage is achieved by extended interactions between one side of the globular A1-domain of vWF and the concave face of the GpIb $\alpha$  N-terminal domain. Two contact sites appear critical in achieving a fine-tuned balance between premature vWF-mediated platelet aggregation in the circulation and lack of binding to immobilised vWF blocking efficient platelet adhesion. The  $\beta$ -switch of GpIb $\alpha$  forms a  $\beta$ -hairpin upon complex formation that aligns with the central  $\beta$ -sheet of vWF-A1. Mutations in the  $\beta$ -switch that increase the  $\beta$ -sheet propensity disturb the balance in the direction of enhanced binding, causing platelet-type pseudo-von Willebrand disease. The N-terminal  $\beta$ -finger of GpIb $\alpha$  contacts A1 on the lower face of the domain, where the gain-of-function mutations related to type 2B von Willebrand disease are clustered, which likely influence the interactions between the A1 domain and its N- and C-terminal flanking peptides. It is possible that shear stress affects this site of A1 in immobilised vWF causing activation in the platelet adhesion process. For treating or preventing arterial thrombosis the interactions between A1 and GpIb $\alpha$  must be destabilized. Our data indicates molecular regions that can be targeted for development of intervening molecules.

## Methods

**Protein expression and purification.** GpIb $\alpha$  residues 1 to 269 and 1 to 290 preceded by the signal peptide and fused to a C-terminal (His) $_6$  or Arg-(His) $_6$  sequence, respectively, were cloned into expression vector pCDNA3.1. The quickchange kit from Stratagene was used to introduce mutations N21Q and N159Q, removing two N-glycosylation sites, and mutation M239V, a platelet-type von Willebrand disease mutation. Proteins were expressed in stable BHK cell-lines. BHK cells were cultured in Dulbecco's MEM/ F-12 Ham medium containing 5% fetal calf serum. During protein production serum was replaced by 1% Ultrosor G (Gibco). GpIb $\alpha$  fragments were purified from expression medium by Ni $^{2+}$ /NTA chromatography, followed by anion exchange (MonoQ)

and gel filtration (Superdex 200). The protein was concentrated to ~7 mg/ml in the gel filtration buffer (50 mM NaCl, 20 mM Tris/HCl pH 8.0). Anion exchange of wild-type and mutant GpIb $\alpha$ (1-290) proteins yielded four base-line separated peaks. Electro-spray mass spectroscopic analysis showed that these peaks contain GpIb $\alpha$  modified by sulfation at 0, 1, 2 or 3 sites, likely tyrosine residues in the anionic region (data not shown). Fully sulphated GpIb $\alpha$  was used for crystallisation experiments.

Wild-type vWF-A1 domain residues 498 to 705 and mutant A1 R543Q were cloned in expression vector pPIC9 and over-expressed in *Pichia pastoris* strain GS115, according to the Invitrogen manual. After 3 days of induction expression medium was collected and dialysed against standard buffer (25 mM Tris, 100 mM NaCl, pH 7.8). The protein was purified on heparin Sepharose, followed by gel filtration (Superdex 200). It was dialysed against standard buffer and concentrated to ~4 mg ml<sup>-1</sup>.

**Crystallisation.** Crystals were grown using the hanging-drop vapour diffusion technique. GpIb $\alpha$  (N21Q, N159Q) crystals were obtained at 28 °C by mixing 1  $\mu$ l of protein (7 mg ml<sup>-1</sup>) and 1  $\mu$ l reservoir solution (1.8 M ammonium sulphate, 0.2 M lithium sulphate and 100 mM CAPS pH 8.2). Before flash freezing, crystals were transferred to a cryo-protective solution (25% (w/v) PEG 3000, 200 mM NaCl, 100 mM Tris pH 8.2 and 15% (v/v) glycerol). GpIb $\alpha$  crystals have space group *C*2 with cell constants:  $a = 121.5 \text{ \AA}$ ,  $b = 54.5 \text{ \AA}$ ,  $c = 101.8 \text{ \AA}$ ,  $\beta = 103.7^\circ$ , and contain two molecules per asymmetric unit.

Crystals of a complex of GpIb $\alpha$  mutant (N21Q, N159Q, M239V) and A1 mutant (R543Q) were obtained at 4 °C by mixing 1  $\mu$ l protein solution (7 mg ml<sup>-1</sup>) containing a 1:1 molar ratio of A1 and GpIb and 1  $\mu$ l precipitant solution (10% (w/v) PEG 3000, 200 mM NaCl and 100 mM MES pH 5.5). Before flash freezing crystals were transferred to precipitant solution containing 20 % (v/v)

glycerol. Crystals have space group  $P6_1$  with cell constants:  $a = b = 89.8 \text{ \AA}$ , and  $c = 124.6 \text{ \AA}$ , and contain one complex per asymmetric unit.

**Structure determination and refinement.** We collected diffraction data of the GpIb $\alpha$ -A1 complex at the X11 beam-line of the EMBL outstation at the  
5 DESY synchrotron in Hamburg and data of GpIb $\alpha$  at beam-line ID 14-2 of the ESRF, Grenoble (Table 1). Data were processed with DENZO and SCALEPACK software. Structures of GpIb $\alpha$  and the GpIb $\alpha$ -A1 complex were solved in conjunction. Molecular replacement with CNS placed A1 in the unit cell of the complex. After solvent flattening with CNS  $\beta$ -strands of GpIb $\alpha$   
10 leucine-rich repeats were clearly resolved. A mask was constructed around the putative GpIb $\alpha$  molecule. Electron density inside the mask was used for molecular replacement with AMORE. This identified two GpIb $\alpha$  molecules in the asymmetric unit of the GpIb $\alpha$  crystal. After refinement of the non-crystallographic symmetry operator, electron density was improved by two-fold  
15 averaging, solvent flattening and phase extension to  $2.5 \text{ \AA}$ . A model was build in the  $2.5 \text{ \AA}$  map with O and later refined at  $1.8 \text{ \AA}$  resolution using CNS. The refined model of GpIb $\alpha$  together with A1 was then used as the starting point for refinement of the GpIb $\alpha$ -A1 complex to a resolution of  $3.1 \text{ \AA}$ .

**Biacore analysis.** Binding studies were performed on a Biacore 2000  
20 (Biacore AB, Uppsala Sweden). GpIb $\alpha$  monoclonal antibody 2D4 was immobilised on CM5-sensorchips by amine-coupling as instructed by the supplier. A control channel was activated and blocked by using the amine-coupling reagents in the absence of protein. Proteins were dialysed to standard Biacore buffer (150 mM NaCl, 0.005% (v/v) Tween-20 and 25 mM HEPES pH  
25 7.4) and analysed at  $25^\circ\text{C}$ . GpIb $\alpha$  (150 nM) was injected for 1 minute, followed by a 2 minute association phase of A1 (5-100 nM) and a 5-minute dissociation phase, during which standard buffer was injected. The sensor-chip was regenerated by first injecting 50 mM triethylamine then 10 mM sodium

formate pH 2.0 and 150 mM NaCl and finally another injection of 50 mM triethylamine. Each run was performed in triplicate. Data evaluation was performed with Bia evaluation software (Biacore AB) using a 1:1 Langmuir model with baseline drift to compensate for the slow release of GpIb $\alpha$  from

5 2D4. Control experiments included immobilisation of GpIb $\alpha$  *via* His-tag antibody 3D5 (Novagen) which excluded artefacts caused by antibody 2D4 and a comparison of glycosylated and non-glycosylated mutant GpIb $\alpha$  to confirm that glycosylation has no effect on binding.

Table 1: Data collection and refinement statistics

Crystal	GpIb $\alpha$	GpIb $\alpha$ A1
Resolution (Å)	1.85/1.9-1.85	3.1/3.2-3.1
Completeness (%)	97.8/80.8	99.9/99.9
Mosaicity (°)	0.4	0.2
Redundancy	3.6/2.4	5.8/5.4
R <sub>merge</sub> (%)	7.3/33.7	8.7/48.0
I/ $\sigma$ I	16.3/2.9	19.3/3.6
R <sub>factor</sub> (%)	19.0	25.1
R <sub>free</sub> (%)	22.0	29.7
No. of protein atoms	4113 (dimer)	3667
No. of waters	638	0
r.m.s.d. bonds (Å)	0.006	0.009
r.m.s.d. angles (°)	1.4	1.7

Table 2: Biacore data

	Dissociation constant (standard deviation) (nM)	
	GpIb $\alpha$ 1-290	GpIb $\alpha$ 1-269
GpIb $\alpha$ (wt)+A1(wt)	33.3 (14.0)	38.4 (14.4)
GpIb $\alpha$ (wt)+A1(mt)	13.6 (3.5)	16.5 (3.14)
GpIb $\alpha$ (mt)+A1(wt)	11.1 (2.1)	12.6 (2.7)
GpIb $\alpha$ (mt)+A1(mt)	5.8 (1.6)	7.5 (2.2)

Figure 1: Structures of the vWF-binding domain of GpIb $\alpha$  and the complex of GpIb $\alpha$  with the A1 domain of vWF.

- a, Ribbon representation of GpIb $\alpha$ . The N-terminal  $\beta$ -hairpin, called ' $\beta$ -finger', is coloured blue, the eight leucine-rich repeats are green and the C-terminal flanking region is red. Disulphide bridges are indicated in yellow ball-and-stick representation. The C-terminal flank contains an exposed loop (residues 227-242), called ' $\beta$ -switch', which is disordered in the uncomplexed structure. b, Stereo view of a ribbon representation of the complex GpIb $\alpha$ -A1. GpIb $\alpha$  is shown in green and A1 in pale blue with mutations GpIb $\alpha$  M239V and A1 R543Q shown in red ball-and-stick representation. The structure reveals an extended site of interactions in which the leucine-rich repeat curve folds around one side of the A1 domain. In the complex the  $\beta$ -switch of GpIb $\alpha$  adopts a  $\beta$ -hairpin structure that aligns with the central  $\beta$ -sheet of A1. c, Representation of residues at the A1- GpIb $\alpha$  interface. Residues involved in inter-molecular contacts shorter than 4.0 Å are shown in stick representation. Residues of GpIb $\alpha$  from leucine-rich repeats 4 to 8 (Val104, Glu128, Lys152, Asp175, Thr176, Pro198 and Phe199) and the C-terminal flank (Glu225, Asn226, Tyr228 and Ser241) form a continuous surface that interacts with A1 residues Glu596, Lys599, Phe603, Gln604 and Ser607 located in helix  $\alpha$ 3 and loop  $\alpha$ 3 $\beta$ 4. Residues Lys8, Ser11, His12, Glu14 and Asn16 of the  $\beta$ -finger and His37 of IIR-1 of GpIb $\alpha$  interact with residues Trp550, Arg571 and Glu613 located in loops  $\alpha$ 1 $\beta$ 2,  $\beta$ 3 $\alpha$ 2, and  $\alpha$ 3 $\beta$ 4 in A1. Leucine-rich repeats 1 to 4 of GpIb $\alpha$  shows no, or few, short contacts with A1. d, Close-up of the  $\beta$ -switch and its interaction with strand  $\beta$ 3 of A1. Main-chain hydrogen bonds are shown by dotted lines. Amino acids known to have gain-of-function mutations related to platelet-type von Willebrand disease have red labels and likely induce  $\beta$ -hairpin formation in the  $\beta$ -switch of GpIb $\alpha$ .

Figure 2: Loss- and gain-of-function mutations in A1 and GpIb $\alpha$ . Mutations shown have been identified by site directed mutagenesis or were detected in patients suffering from four different bleeding disorders. **a**, Loss-of-function mutations. Mutations in GpIb $\alpha$  causing Bernard-Soulier syndrome are shown in purple, mutations in A1 related to type 2M von Willebrand disease are shown in red ball-and-stick representation. **b**, Gain-of-function mutations. Mutations in GpIb $\alpha$  causing a platelet type von Willebrand disease phenotype are shown in purple, mutations in A1 related to type 2B von Willebrand disease are shown in red ball-and-stick representation. The A1-domain and GpIb $\alpha$  are shown in blue and green ribbon representation, respectively

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### Claims

1. A method to interfere with platelet adhesion comprising modulating the adhesion of amino acid residues at amino acid position 560-566 of strand  $\beta 3$  of domain A1 of von Willebrand factor (vWF) to amino acid residues at amino acid position 227-242 of a region of platelet GpIb $\alpha$ .
- 5 2. A method to interfere with platelet adhesion comprising modulating the adhesion of amino acid residues at amino acid position 500-600 of strand  $\beta 3$  of domain A1 of von Willebrand factor (vWF) to amino acid residues at amino acid position 200-300 of a region of platelet GpIb $\alpha$ .
- 10 3. A chemical or proteinaceous compound capable of interfering with the binding of amino acid residues at amino acid position 560-566 of strand  $\beta 3$  of domain A1 of von Willebrand factor (vWF) to amino acid residues at amino acid position 227-242 of a region of platelet GpIb $\alpha$ .
- 15 4. A proteinaceous compound according to claim 3, comprising an amino acid sequence corresponding to amino acid position 560-566 of strand  $\beta 3$  of domain A1 of vWF and/or a functional part or equivalent thereof.
5. A proteinaceous compound according to claim 3, comprising an amino acid sequence corresponding to amino acid residues at amino acid position 227-242 of platelet GpIb $\alpha$  and/or a functional part or equivalent thereof.
- 20 6. A compound according to claim 3, comprising an antibody and/or a functional part or equivalent thereof, specifically recognising said region of platelet GpIb $\alpha$  and/or said strand  $\beta 3$  of domain A1 of vWF
7. Use of a compound according to any of claims 3 -6, for interfering with platelet adhesion and/or thrombus formation.
- 25 8. Use of a compound according to any of claims 3-6, wherein said compound is linked to another chemical and/or proteinaceous compound, wherein said second compound modulates the circulation time of the first compound.

9. Use of a compound according to any of claims 3-6 for the preparation of a medicament for the treatment or prevention of a disease in which platelet adhesion and/or thrombus formation is involved.
10. A pharmaceutical composition for inhibiting platelet adhesion and/or thrombus formation comprising a compound according to any of claims 3-6, further comprising a suitable carrier or solvent.
11. Use of a medicament according to claim 9, or a pharmaceutical composition according to claim 10, to modulate platelet adhesion and/or thrombus formation .
12. An antibody and/or a functional part or equivalent thereof, specifically recognising a region of platelet GpIb $\alpha$  according to claim 1, capable of interfering with platelet adhesion.
13. An antibody and/or a functional part or equivalent thereof, specifically recognising strand  $\beta$ 3 of domain A1 of vWF, capable of interfering with platelet adhesion.
14. Use of an antibody and/or a functional part or equivalent thereof according to claim 12 or 13 for the preparation of a medicament for the treatment of a disease in which platelet adhesion and/or blood clotting is involved.
15. A vector which is capable of effecting the expression of a peptide with an amino acid sequence corresponding to a beta-switch loop of platelet GpIb $\alpha$ , comprising amino acid residues at amino acid position 227-242 and/or a functional part or equivalent thereof, in an appropriate environment.
16. A vector which is capable of effecting the expression of a peptide with an amino acid sequence corresponding to strand  $\beta$ 3 of domain A1 of vWF, comprising amino acid residues at amino acid position 560-566 and/or a functional part or equivalent thereof, in an appropriate environment.
17. A method of treatment or prevention of a disease condition in which platelet adhesion is involved, comprising administering a medicament

according to claim 9 and/or a pharmaceutical composition according to claim 10.

18. A method of treatment according to claim 17, comprising providing an antibody according to claim 12 and/or 13, and or a vector according to claim 15 and/or 16.

19. A method to enhance platelet adhesion comprising local application of a peptide or a proteinaceous compound with an amino acid sequence corresponding to strand  $\beta 3$  of domain A1 of vWF, comprising amino acid residues at amino acid position 560-566 and/or a functional part or equivalent thereof, capable of binding platelet GpIb $\alpha$ .

20. Use of a peptide or a proteinaceous compound with an amino acid sequence corresponding to strand  $\beta 3$  of domain A1 of vWF, comprising amino acid residues at amino acid position 560-566 and/or a functional part or equivalent thereof, for the manufacturing of a topical medicament for local enhancement of platelet adhesion and/or thrombus formation.

21. A pharmaceutical composition for local enhancement of platelet adhesion and/or thrombus formation comprising a peptide or a proteinaceous compound with an amino acid sequence corresponding to strand  $\beta 3$  of domain A1 of vWF, comprising amino acid residues at amino acid position 560-566 and/or a functional part or equivalent thereof, and a suitable carrier or solvent.

22. A method of treatment or prevention of a disease condition in which platelet adhesion is decreased for example, but not limited to von Willebrand disease type 2M, comprising providing a medicament according to claim 20 and/or a pharmaceutical composition according to claim 21.

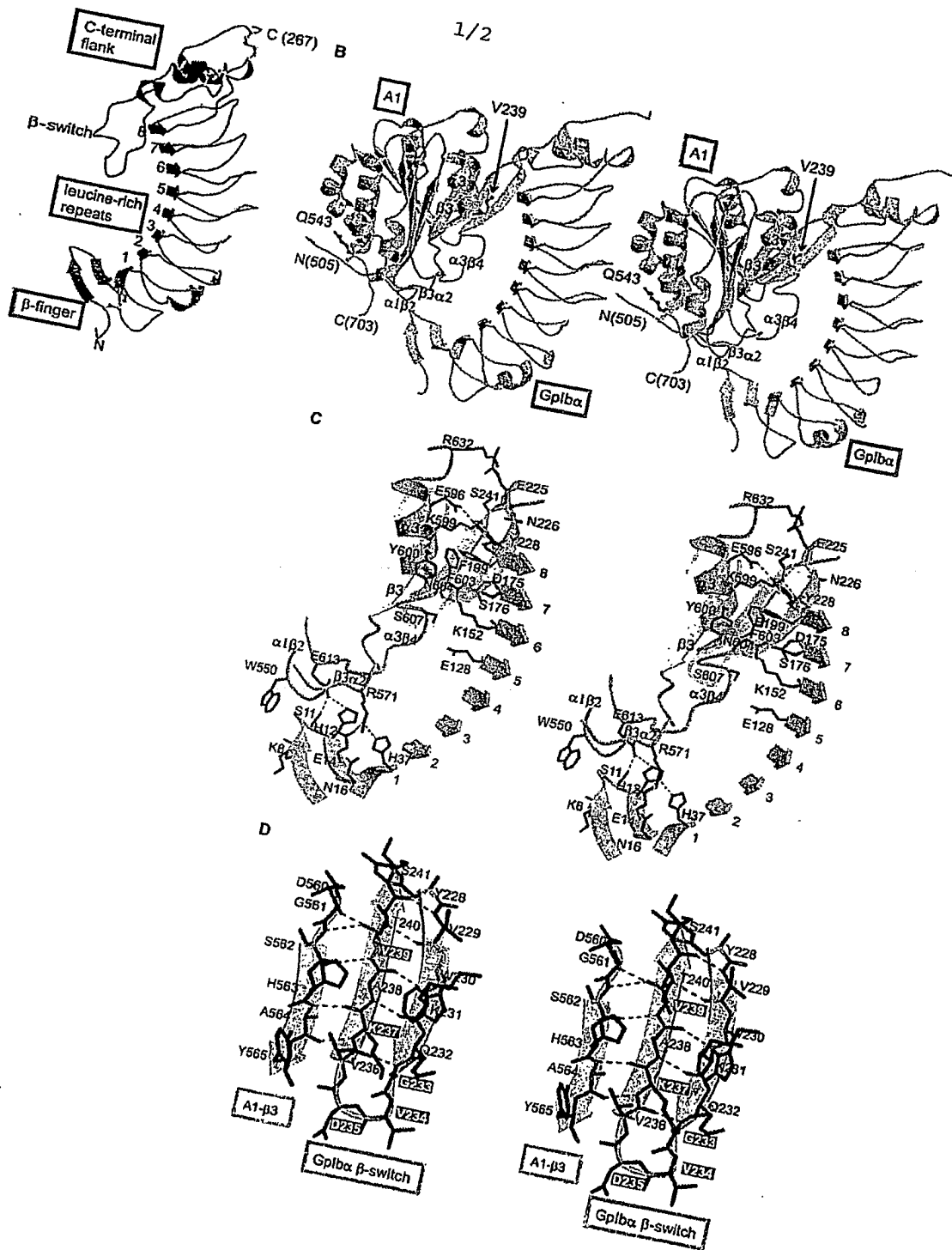


Fig. 1

2/2

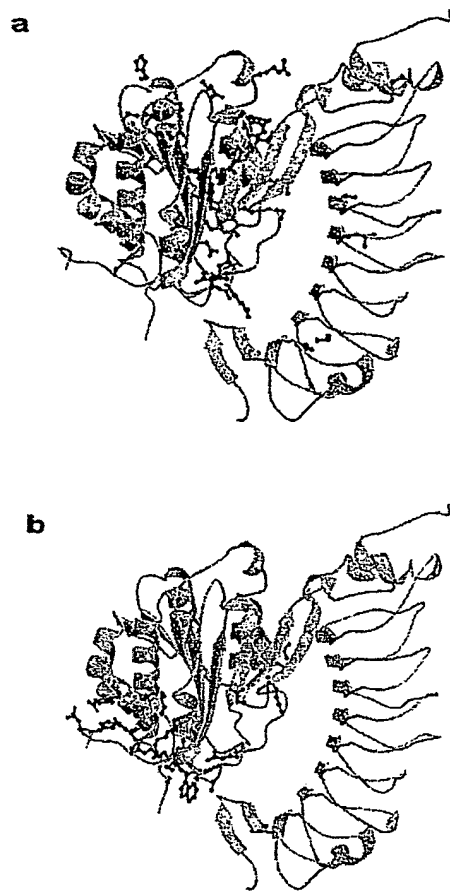


Fig. 2.

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/NL 03/00564

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 7 G01N33/68 C07K14/47 C07K14/755

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 7 G01N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DONG JING-FEI ET AL: "Novel gain-of-function mutations of platelet glycoprotein Ibalpha by valine mutagenesis in the Cys209-Cys248 disulfide loop: Functional analysis under static and dynamic conditions." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 275, no. 36, 8 September 2000 (2000-09-08), pages 27663-27670, XP002226851 ISSN: 0021-9258 in particular abstract and p.27669, left column, last paragraph the whole document --- -/--	1,2

☒ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

### \* Special categories of cited documents :

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \*Z\* document member of the same patent family

Date of the actual completion of the international search

20 October 2003

Date of mailing of the international search report

27/10/2003

Name and mailing address of the ISA  
European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Lüdemann, S

## INTERNATIONAL SEARCH REPORT

Internat  
PCT/NL 03/00564

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	VASUDEVAN SONA ET AL: "Modeling and functional analysis of the interaction between von Willebrand factor A1 domain and glycoprotein Ibalpha." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 275, no. 17, 28 April 2000 (2000-04-28), pages 12763-12768, XP002226852 ISSN: 0021-9258 in particular tab.1 and fig. 2 the whole document	1,2
Y	CRUZ MIGUEL A ET AL: "Mapping the glycoprotein Ib-binding site in the von Willebrand factor A1 domain." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 275, no. 25, 23 June 2000 (2000-06-23), pages 19098-19105, XP002226853 ISSN: 0021-9258 in particular fig. 6 the whole document	1,2
T	HUIZINGA ERIC G ET AL: "Structures of glycoprotein Ibalpha and its complex with von Willebrand factor A1 domain." SCIENCE (WASHINGTON D C), vol. 297, no. 5584, 2002, pages 1176-1179, XP002226858 16 August, 2002 ISSN: 0036-8075 the whole document	1,2

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/NL 03/00564

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 17-19 and 22  
because they relate to subject matter not required to be searched by this Authority, namely:  
Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy
2. ☒ Claims Nos.: 3-22  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:  
see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

## FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 3-22

Present claims 3, 6-14, 17 and 18 relate to a product/method defined by reference to a desirable characteristic or property, namely "capable of interfering with the binding of amino acid residues at ..." or "specifically recognising...".

The claims cover all products/methods having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and disclosure within the meaning of Article 5 PCT for none of such products/methods. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT).

Claims 4, 5, 15, 16, 19-22 relate to a proteinaceous compound for which the sequence listing has NOT been provided according to R. 13 ter. The subject-matter of said claims can therefore not be searched.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.